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Sequence of subunit *c* of the Na^+-translocating F_1F_O ATPase of *Acetobacterium woodii*: proposal for determinants of Na^+ specificity as revealed by sequence comparisons

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Abstract A 3.2 kb *Eco*RI fragment carrying genes for Na^+-F_1F_0 ATPase was cloned from chromosomal DNA of *Acetobacterium woodii*. DNA sequence analysis revealed the presence of an open reading frame which was identified by data base searches and comparison with the experimentally derived N-terminal amino acid sequence to code for subunit *c* of Na^+-F_1F_0 ATPase. A comparison of the primary sequences of the two well established Na^+-translocating F_1F_0 ATPases from *Acetobacterium woodii* and *Propionigenium modestum* with H^+-translocating enzymes indicates the length of the C-terminus as well as specific residues located in the cytoplasmic membrane to be important for Na^+ transport.

Key words: ATPase; Na^+-F_1F_O; Subunit *c*; DNA sequence; *Acetobacterium woodii*

1. Introduction

F_1F_O ATPases are reversible enzymes which couple ATP synthesis in the F_1 domain with the flow of ions through the F_O domain [1,2]. Although the enzymes have been known for decades, the mechanism of ion transport as well as the coupling of ATP synthesis with ion flow is far from being settled. An alternative approach to elucidate their function is to perform a comparative study with enzymes exhibiting an altered ion specificity. The anaerobic bacteria *Propionigenium modestum* and *Acetobacterium woodii* are the only well established examples of organisms having ATPases which, as revealed by inhibitor studies, subunit composition, primary sequences and electron microscopy, are typical F_1F_O enzymes but use Na^+ instead of H^+ as coupling ion [3–5]. The F_O complex was shown to determine the ion specificity of the enzyme [6] and the 'active carboxylate' of subunit *c* was shown biochemically to contribute to Na^+ liganding [7,8]. As a first step towards a better understanding of Na^+ transport through the F_1F_O ATPase from *A. woodii* we cloned and sequenced the gene encoding subunit *c*. This sequence is now the second sequence available for subunit *c* from Na^+-F_1F_O ATPases. A comparison of these two sequences to those from H^+-F_1F_O ATPases revealed determinants probably responsible for Na^+ specificity.

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2. Materials and methods

2.1. Organism and plasmids

A. woodii (DSM 1030) was obtained from the Deutsche Sammlung für Mikro-organismen und Zellkulturen, Braunschweig, Germany, and grown under strictly anaerobic conditions on carbonate-buffered medium supplied with 0.4% glycine [9]. *E. coli* DH5α (*supE*44 Δ*lac*U169(Φ80*lacZ*ΔM15) *hsdr*17 *recA*1 *endA*1 *gyrA*96 *thi*1 *relA*1 [10]) was grown on LB at 37°C. Plasmids used were pSE420 [11], pHSG 398 and 399 [12], and pBluescript II SK and KS (Stratagene, San Diego, CA, USA).

2.2. Molecular procedures

Chromosomal DNA of *A. woodii* was isolated by a modified Marmur preparation [13]. Cells were grown to late logarithmic growth phase, pelleted by centrifugation and lysed by alkaline SDS solution. The protein was precipitated with 6 M guanidinium thiocyanate and isobutanol, and the DNA was bound to glasmilk in the presence of NaJ (90.8 g NaJ, 1.5 g Na_2SO_3, H_2O ad 100 ml). After washing with ethanol (50%), NaCl (0.1 M), Tris-HCl (0.02 M, pH 7.5) and EDTA (1 mM), the DNA was eluted with TE buffer. The DNA was then restricted, size fractionated by gradient centrifugation and cloned into pSE420. All procedures used were standard techniques [10]. Oligonucleotides were synthesized on a Gene Assembler Plus apparatus as recommended by the manufacturer (Pharmacia LKB). DNA sequence was determined by the chain termination method of Sanger and analyzed on a VAX computer using the Wisconsin genetics computer group sequence analysis software package, version 8.1 (University of Wisconsin Biotechnology Center, Madison, WI, USA).

3. Results and discussion

A homologous probe covering the DNA region upstream of the *Eco*RI site in *atpA* [5] was amplified by PCR with oligonucleotides derived from the known DNA sequence in *atpA* and from the N-terminal amino acid sequence of the 19 kDa subunit. Southern blot analysis of genomic DNA restricted with *Eco*RI revealed a 3.2 kb fragment that hybridized with the homologous probe. This fragment was cloned in pSK^+, designated pSR4 and partially sequenced.

At the 5′ end of pSR4 an open reading frame of 270 bp was found. This ORF was identified by data base searches as *atpE* which encodes subunit *c* of F_1F_O ATPases. The identity of the gene was verified by comparison of the deduced amino acid sequence which matched the experimentally derived N-terminal sequence of subunit *c* of the Na^+-F_1F_O ATPase from *A. woodii* [4]. *atpE* starts with an ATG codon and encodes a strongly hydrophobic protein with a molecular mass of 8.96 kDa (Fig. 1). 60, 47, and 23% of its residues are identical with subunit *c* from the Na^+-F_1F_O ATPase from *P. modestum*, the H^+-F_1F_O ATPase from *Synechocystis* 6803 and the H^+-F_1F_O ATPase from *Escherichia coli*, respectively.

A comparison of the deduced amino acid sequences with H^+-translocating F_1F_O ATPases revealed the following un-

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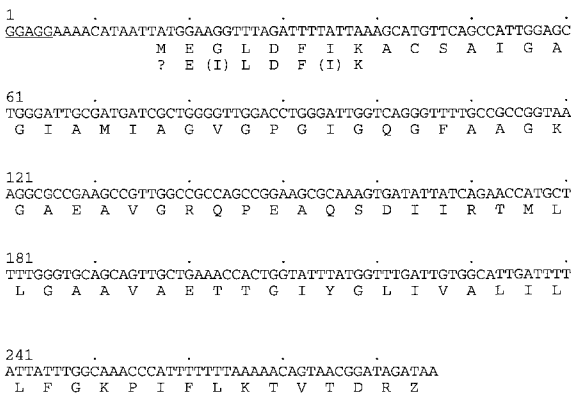

Fig. 1. Nucleotide sequence and deduced amino acid sequence of *atpE* of *A. woodii*. The sequence was obtained by primer walking. Shine-Dalgarno sequences are underlined, and the experimentally derived N-terminal amino acid sequence is given below the translated sequence. The sequence has been submitted to GenBank and is available under accession number U63995.

usual features in subunit *c* from *A. woodii* and *P. modestum* which we propose are relevant for Na^+ transport:

(1) Subunit *c* folds in the membrane like a hairpin with its N- and C-termini being located in the periplasm [14]. Interestingly, the size of subunit *c* in *A. woodii* is increased by two residues at the N-terminus and, more important, nine residues at the C-terminus; this enlarges the hydrophilic, periplasmic portion of the polypeptide (Fig. 2). As expected, such an increase is also observed with the Na^+ ATPase from *P. modestum*. Therefore, this feature can be considered significant in determining the ion specificity. As discussed before for *P. modestum* [15], the enlargement of subunit *c* might alter the geometry of the F_O complex facing the periplasm, by altering the interaction either of multiple copies of subunit *c* or of subunit *c* with other membrane-integral subunits. Taking into consideration the presence of a selectivity filter at the mouth of the F_O complex, as envisaged by Fillingame [16], the additional residues in subunit *c* of *P. modestum* and *A. woodii* might constitute (or contribute to) such a filter which widens the mouth and allows the passage of the large sodium ion into the F_O complex. This assumption is corroborated by the finding that random mutagenesis of subunit *c* of *P. modestum* led to mutants which lost Na^+ but retained H^+ transport by exchange of the bulky residues F84 and L87 against smaller residues [15]. Based on these findings we predict that deletion of the periplasmic C-terminus of *A. woodii* and *P. modestum* would result in a loss of Na^+ with a retention of H^+ pumping.

(2) Residues able to ligand the Na^+ ion should be present in Na^+ but not H^+ ATPases. In the membrane spanning regions of subunit *c* of *A. woodii* six residues are present which have the potential ability to ligand ions by use of their free electron pair: M19, P25, Q29, E62, T63 and T64. A multiple alignment revealed that only P25, Q29, E62, T63 and T64 are conserved or functionally substituted in Na^+-F_1F_O ATPases from *A. woodii* and *P. modestum* and in the second half of subunit *c* of the Na^+-V_1V_O ATPase from *Enterococcus hirae* but not in H^+-translocating F_1F_O ATPases (Fig. 2). E62 and T63 but not T64 were shown by site directed mutagenesis to be essential for Li^+ binding in the F_1F_O ATPase from *E. coli* [17]. Therefore, we predict that P25, Q29, E62 and T63 constitute (or contribute to) the Na^+ binding site of the Na^+ ATPase of *A. woodii*. These four residues are located at approximately the same distance from the surface of the cytoplasmic membrane, and therefore would also physically be able to build at least part of the cavity in which the Na^+ ion is coordinated (Fig. 3). Experimental evidence for a close proximity of A24 and I28 of helix one (corresponding to P25 and Q29 in *A. woodii*) to D61 of helix two (corresponding to E62 in *A. woodii*) in *E. coli* is available [18,19].

In conclusion, the data available now revealed two determinants for Na^+ specificity of the F_1F_O ATPase of *A. woodii*: an enlargement of the C-terminus of subunit *c* accompanied by a change in the geometry of the complex and the presence of the sodium ion binding motif P25, Q29, E62 and T63; residues in this motif can be substituted by functionally identical residues, i.e. those of appropriate size and bearing a free electron pair. Although one or the other determinant is

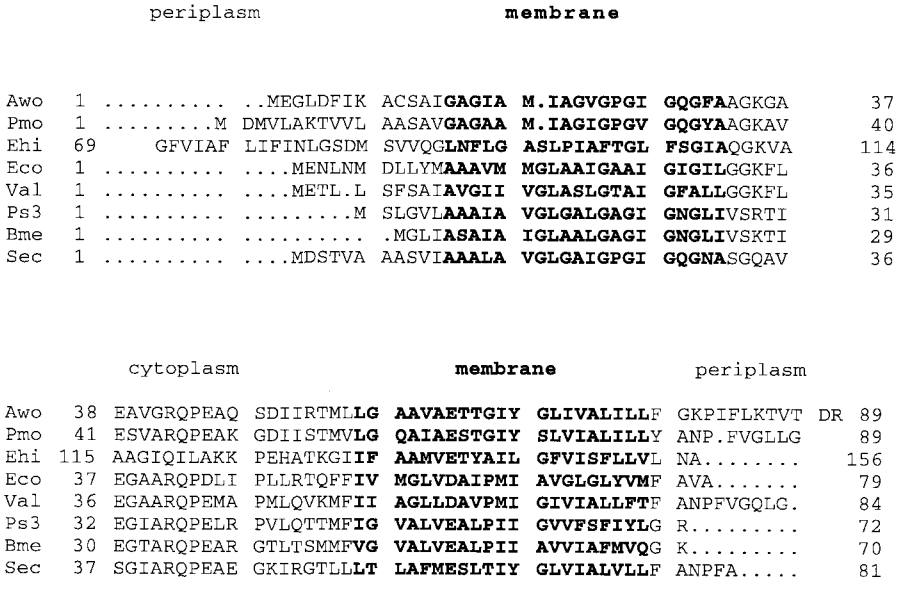

Fig. 2. Alignment of subunit *c* from *A. woodii* with subunit *c* from *P. modestum* [20], *E. hirae* [21], *E. coli* [22], *V. alginolyticus* [23], thermophilic bacterium PS3 [24], *Bacillus megaterium* [25], and *Synechocystis* 6803 [26]. The topology of subunit *c* is indicated.

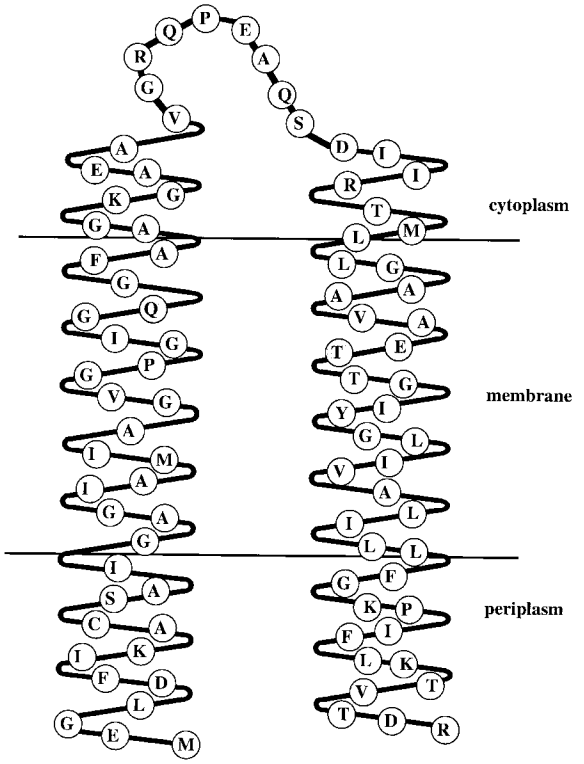

Fig. 3. Hairpin folding model of subunit *c* of the Na^+-F_1F_O ATPase of *A. woodii*. A potential sodium ion binding motif is indicated.

present in certain other enzymes (i.e. an enlarged C-terminus in *V. alginolyticus* and a sodium ion binding motif in *Synechocystis* 6803), both are present only in Na^+-F_1F_O ATPases from *A. woodii* and *P. modestum*.

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